IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of A. K. Gunnar Aberg and George E. Wright and Jan L. Chen and Andrew T. Maioli

Application No.: 10/069,663 Filing Date: 02/27/2002

For: Optically Active Isomers of Ketotifen and Therapeutically Active Metabolites Thereof

DECLARATION UNDER 37 C.F.R. § 1.132

The Honorable Commissioner
Of Patents & Trademarks
Washington, D.C. 20231

Sir:

I, A.K. Gunnar Aberg, declare:

THAT I am a citizen of Sweden, a permanent resident of the USA, and resident of the City of Sarasota, Sarasota County, Florida;

THAT I am now the Chief Executive Officer of BRIDGE PHARMA, Inc., 902 Contento Street, Sarasota, Florida 34242. From 1968 to 1973, I was Director of Pharmacology at Bofors Nobel-Pharma (Sweden); from 1974 to 1978, I was Group Leader in General Pharmacology at AB Hässle (Sweden); from 1978 to 1980, I was Director of Pharmacology at Astra (USA); from 1980 to 1982, I was Director of Cardiovascular Pharmacology at Ciba-Geigy (USA); from 1982 to 1992, I was Director and Executive Director of Pharmacology at Squibb and Bristol-Myers Squibb; and from 1992 to 1996, I was Vice President and Senior Vice President of Research at Sepracor Inc.;

THAT I am a graduate of the University of Linköping, Sweden from which I hold a Ph.D. degree in Pharmacology and of the University of Gothenburg, Sweden from which I hold a degree in Zoophysiology, and that I am a docent (Associate Professor) in Applied Pharmacology at the University of Linköping, Sweden;

THAT I have over thirty years of industrial experience of pharmacological research;

THAT I am an author of over one hundred publications on pharmacological and toxicological topics, including eighteen publications and abstracts on drugs that are used in asthma;

THAT my Ph.D. thesis in pharmacology concerned pharmacological effects of optically active isomers and that forty of my publications concern biological activities of optically active isomers of various drugs;

THAT I am an inventor of approximately 45 U.S. patents and a number of pending patent applications, including the present Patent Application;

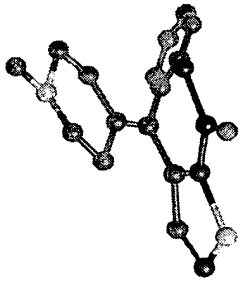
THAT I have reviewed the Office Action dated April 07, 2003 in the above referenced Application. I am also familiar with the application in the present case and the art cited by the Examiner, namely Polivka et al. Czech Patent 263993 (1998) and Polivka et al., Collect. Czech. Chem. Commun. 1989, 54: 2443 – 2469, Le Bigot et al. Drug Metab. Dispos. 1983, 11: 585 – 589, Le Bigot et al., Life Sci.,1987, 40: 883 – 390, Bourquin et al., (1972) US Patent 4,128,156 and Kofler et al., Jap Chem Soc (1957),1:240 – 260.

THAT biological studies of compounds of the present application have been designed by me and performed under my close supervision. The study results support the subject application. A summary of the studies will follow.

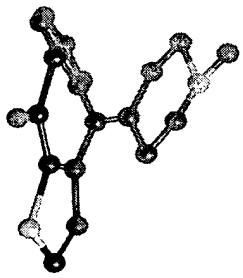
This report contains a summary of all studies known to me on the isomers of ketotifen (pages 2 to 5) and a summary of our own studies (pages 6 to 21) that concern the racemates and the isomers of ketotifen and norketotifen.

Isomers of ketotifen and norketotifen.

Polivka et al. (1989) first reported that ketotifen exists in optically active forms despite its lack of chiral carbons. The chirality results from restricted rotation of the piperidylene ring, which gives rise to perpendicular dissymmetric planes. This is also referred to as atropisomerism, and the two enantiomers (mirror images or optical antipodes) are referred to as atropisomers. For example, ketotifen is not planar, as there exists a twist in the structure (shown below, adapted from the coordinates of the X-ray crystal structure of R-ketotifen dibenzoyl D tartaric acid; Polivka et al. 1989). The importance of the N-methyl substituent for optical stability of the ketotifen atropisomers was unknown at the onset of our project. There is apparently a high energy barrier to chemical interconversion of the ketotifen atropisomers as racemization proceeds at the melting point of the bases (158 - 160°C) and in boiling aqueous solutions of the salts (Polivka, 1989). However, in general, the energy barrier for chemical interconversion of atropisomers as well as the in vivo conditions for metabolic interconversion may vary widely according to the molecular structure and so there is no rule of thumb.



S-Ketotifen



R-Ketotifen

Although atropisomerism is a known form of chirality, its occurrence in drug candidates and pharmaceuticals is uncommon, but not unprecedented. Notably, and in addition to ketotifen, the natural product and antibiotic of last resort, vancomycin, is chiral as a result of atropisomerism.

Purpose of Project.

Ketotifen was introduced as an antihistaminic drug by Sandoz about 30 years ago. Since the drug was significantly more sedating than diphenhydramine (Benadryl®), ketotifen was not introduced in the USA as an orally active antihistamine. About 15 years ago it was found in Japan that ketotifen had anti-inflammatory effects in addition to the known antihistaminic activity. However, due to the severe sedative side effects of the drug, the daily dose was limited to 1 mg orally, twice daily. This dose was too low to offer a reliable and potent therapeutic effect to patient suffering from asthma, atopic dermatitis and other inflammatory diseases. The purpose of the present project has been to reduce – or if possible, eliminate – the sedative side effects of ketotifen, while retaining the therapeutic activity of the drug.

PHARMACOLOGICAL STUDIES REPORTED BY POLIVKA

The only pharmacological studies found by us on the isomers of ketotifen are those summarized Polivka et al., Czech Pat. 263993 (herein called Polivka I) and Polivka et al., Collect Czech Commun 1989, 54:2443-2469 (herein called Polivka II).

It is important to observe that the optical purity of the test samples of R- and S-ketotifen was not shown.

1. (Polivka I and II). Antihistaminic activity in vivo:

Test articles (R-ketotifen or S-ketotifen) were given orally to guinea pigs that were exposed to histamine aerosol.

	PD_{50}, mg/kg
R(+)-ketotifen	0.026
S(–)-ketotifen	0.013
RS-ketotifen	N/A

Conclusion: These *in vivo* results indicate that S-ketotifen was more active than R-ketotifen with a factor of 2.

2. (Polivka I and II). Dermal activity in 48/80 tests

Test articles (R-ketotifen or S-ketotifen) were given orally to rats that were Given dermal injections of substance 48/80, a well-known histamine-releasing agent.

	PD_{50}, mg/kg	
R(+)-ketotifen	4.2	
S(–)-ketotifen	>50	1 mg/kg → 25%.
RS-ketotifen	N/A	

Conclusion: These *in vivo* results indicate that R-ketotifen was biologically active, while no activity was observed with S-ketotifen.

3; 4. (Polivka I and II). Antihistaminic and antimuscarinic receptor affinities: The affinity for H-1 receptors is described in Polivka II and tests of affinity for muscarinic receptors is described in both Polivka I and Polivka II

	H-1 (IC_{50}, nM)	M (IC_{50}, nM)
R(+)-ketotifen	6.0	149
S(–)-ketotifen	40.0	1,217
RS-ketotifen	10.0	260

Conclusion: These *in vitro* results indicate that R-ketotifen carries most of the anti-histaminic and anti-muscarinic activities, while S-ketotifen was significantly less active. The R-isomer was approximately 6.5 times more active than the S-isomer on H-1 receptors and approximately 8 times more active on the muscarinic receptors.

5. (Polivka II). In vivo antihistaminic activity (s.c. dosing)

The study was performed in rats after intradermal injections of histamine and subcutaneous administration of the test articles.

	ID_{50}, μg/kg
R(+)-ketotifen	0.57
S(–)-ketotifen	3.6

Conclusion: In this *in vivo* antihistamine study, R-ketotifen was reportedly about 6.5 times more active than S-ketotifen.

General conclusion from the Polivka pharmacological studies:

Five pharmacological studies were performed by Polivka et al. The R-isomer of ketotifen was more active (by a factor of at least 6.5) than the S-isomer in four studies (H-1 receptor affinity; M receptor affinity; in vivo s.c. antihistamine activity; 48/80 histamine release) while the S-isomer of ketotifen was more active than the R-isomer (by a factor of 2) in one study (in vivo antihistamine activity after oral administration). Polivka draws the conclusion that R-ketotifen "is evidently the more active ketotifen enantiomer". However, he added, "the stereoselectivity of action is only a partial one" (Polivka I, p 2457.)

Thus, the teaching of Polivka is in the direction of the R-enantiomer of ketotifen being more active than the corresponding S-enantiomer.

CHEMISTRY AND PHARMACOLOGY BY THE PRESENT INVENTORS

A. CHEMISTRY

The synthetic work was performed or directed by the inventors, Chen, Maioli and Wright in collaboration with the co-inventor Aberg.

1. Chemical Synthesis

Racemic ketotifen is commercially available (Sigma).

R(+)- and S(–)-ketotifen were made according to Polivka et al., 1989 (CS 263993)

Racemic norketotifen can be made according to the present patent application or the patent application by Friary et al. that describes $3{,}768 \times 10^{15}$ compounds (89304168.1)

R(+)- and S(–)-norketotifen could not be made according the methodology described by Polivka et al. (CS 263993), but a new synthetic route had to be developed. Thus, the demethylation of S-ketotifen free base was effected in two steps: first, treatment with 2,2,2-trichloroethyl chloroformate produced the trichloroethyl carbamate. The second step, which included the cleavage of the carbamate, without causing racemization proved to be problematic and the existence of stable enantiomers of norketotifen was questioned. However, it was found that cleavage at room temperature with cadmium metal (rather than refluxing aqueous sulfuric acid in ethanol as with the racemate), furnished S-norketotifen in 98.5 % ee. It is our belief that the new conditions are mild and therefore caused very little loss in optical activity. The new synthesis of the norketotifen enantiomers is described and claimed in the present patent application.

2. Chemical and Optical Purity

The chemical and optical purity of the Bridge Pharma samples of R(+)- and S(–)-ketotifen and of R(+)- and S(–)-norketotifen are better than 98 % ee.

3. Enantiomeric Configuration

The absolute configuration of the (+)-enantiomer of ketotifen was determined by Polivka et al. (Collect.Czech. Chem. Commun. 1989, 34: 2443-2469) by X-ray crystallography, and was designed "R" with the conventions described by Cahn et al. (Angew. Chem. Intl. Ed. 1966, 5: 385-415.) Thus the () enantiomer was designated "S". Absolute configurations and optical rotations of the enantiomers are shown in the following table. An third experimental distinction between the enantiomers is their relative mobility in chiral high performance liquid chromatography (HPLC). Our analysis of the enantiomers (using a Cyclobond I 2000 chiral HPLC column) showed that the retention time for R-(+)-ketotifen was longer than for S(–)-ketotifen. Thus we designated the former as "slow" (s) and the latter as "fast" (f) (See table below).

The ketotifen enantiomers were used to prepare the corresponding enantiomers of norketotifen. It should be pointed out that the existence of stable

norketotifen enantiomers was unknown at the onset of these studies, since the importance of the N-methyl group for the chiral stability was unknown for the atropisomers of ketotifen. It was found that the reaction conditions retained the configuration of the original enantiomers, because no sign of loss of optical rotation or loss of enantiomeric excess (by chiral HPLC) were observed. Thus, (+)-ketotifen gave (+)-norketotifen, and (–)-ketotifen gave (–)-norketotifen. In chiral HPLC, the (+)-enantiomer of norketotifen was s and the (–)-enantiomer was f (see table below).

In conclusion, the identity of each enantiomer may be designed in three ways — optical rotation (+ or –), chiral HPLC retention time (f or s), and absolute configuration (R or S). Summary relationships and designations of the enantiomers are shown in the following table.

Compound	Optical Rotation $[a]_D$, EtOH	Retention time Chiral HPLC, min	Absolute configuration
R-(+)-s-ketotifen	+58.9°	8.99	R
S-(–)-f-ketotifen	–61.6°	5.92	S
R-(+)-s-norketotifen	+62.8°	8.13	R
S-(–)-f-norketotifen	–62.6	5.29	S

General Conclusion (Chemistry)

The enantiomeric forms of ketotifen were described in the prior art (Polivka et al.) and have now been synthesized according to the previously described method. The absolute configurations of R- and S-ketotifen in the prior art were confirmed.

The existence of stable norketotifen enantiomers was unknown at the onset of these studies. No methods for making enantiomeric forms of norketotifen were known at the onset of the present studies. The present inventors have developed a method for synthesizing the enantiomeric forms of norketotifen, the inventors have determined that these forms exist as stable chemical entities and the inventors have determined the absolute configuration of the norketotifen enantiomers.

B. PHARMACOLOGY

The pharmacological work was performed or directed by the inventor Aberg, in collaboration with the co-inventors Chen, Maioli and Wright.

1. Affinities for H-1 receptors

(H-1 affinity refers to antihistaminic activity, which is a therapeutic effect)

The affinities of the racemic and isomeric test compounds for the histamine H-1 receptor were assessed using the ^{3}H-pyrilamine binding assay as described by Dini et al. (Agents and Actions, 1991, 33: 181-184). Briefly, membranes from guinea pig cerebellum were incubated with ^{3}H-pyrilamine and varying concentrations of the test compound(s). The specific binding of the radioactive ligand to the receptor was defined as the difference between total binding and nonspecific binding, determined in the presence of an excess of unlabelled ligand. The results were expressed as percentage of specific binding in the presence of compounds. IC_{50} values (concentration required to inhibit 50% of specific binding) were determined by non-linear regression analysis of the competition curves. These parameters were obtained by Hill equation curve fitting using Sigmaplot™ software. The inhibition constants (Ki) were calculated from the Cheng Prusoff equation (Ki = IC50/(1+L/Kd), where L = concentration of radioligand in the assay, and Kd = affinity of the radioligand for the receptor).

Two studies have been performed, one with the ketotifen isomers and one with the norketotifen isomers.

Study 1: Ketotifen Isomers.

	H-1 receptors	
	IC50 (nM)	Ki (nM)
R(+)-ketotifen	20.0	3.2
S(–)-ketotifen	7.6	1.2
RS-ketotifen	10.0	1.6
Pyrilamine (ref.)	2.9	0.46

Conclusion: These *in vitro* results demonstrate that S-ketotifen had higher affinity than R-ketotifen for histamine H-1 receptors.

Study 2: Norketotifen Isomers.

	Peripheral H-1 receptors	
	IC50 (nM)	Ki (nM)
R(+)-norketotifen	134	21
S(–)-norketotifen	90	14
RS-norketotifen	94	15
Pyrilamine (ref.)	2.4	0.38

Conclusions: The results from Studies 1 and 2 demonstrate that racemic ketotifen is more potent than racemic norketotifen as an H-1 antagonist *in vitro*. S-norketotifen had higher affinity than R-norketotifen for histamine H-1 receptors.

2. Affinities for M-1 receptors
(Antimuscarinic M-1 activity causes inhibition of the production of saliva and sweat and causes ocular accommodation difficulties and is an unwanted side-effect)

The experiments were carried out on membranes prepared from SF9 cells infected with baculovirus to express the human recombinant muscarinic M-1 receptor subtype. After incubation with the test article and the proper radioligand and washing, bound radioactivity was determined with a liquid scintillation counter, using a commercial scintillation cocktail. The specific radioligand binding to each receptor was defined as the difference between total binding and nonspecific binding in the presence of an excess of unlabelled ligand. IC_{50} values (concentrations required for 50% inhibition of specific binding) were determined by non linear regression analysis of the competition curves. The inhibition constants (Ki) were calculated from the Cheng Prusoff equation (Ki = IC50/(1+L/Kd), where L = concentration of radioligand in the assay, and Kd = affinity of the radioligand for the receptor).

Two studies have been performed, one with the ketotifen isomers and one with the norketotifen isomers.

Study 1: Ketotifen Isomers.

	Muscarinic M-1 receptors	
	IC50 (nM)	Ki (nM)
R(+)-ketotifen	109	92
S(–)-ketotifen	740	626
RS-ketotifen	305	258
Pirenzepine (ref.)	14	12

Conclusion: The results surprisingly demonstrate that R-ketotifen had higher affinity for the human M-1 receptors than S-ketotifen. Antimuscarinic activity is considered to be a side effect of antihistamines and is poorly tolerated by patients suffering from atopic dermatitis, in whom muscarinic (M-1) inhibition is causing dry skin.

Study 2: Norketotifen Isomers.

	Muscarinic M-1 receptors	
	IC_{50} (nM)	Ki (nM)
R(+)-norketotifen	1070	905
S(–)-norketotifen	3610	3050
RS-norketotifen	1440	1220
Pirenzepine (ref.)	20	17

Conclusion: The results from Studies 1 and 2 surprisingly demonstrate that racemic norketotifen is significantly less active than racemic ketotifen as an M-1 antagonist. Surprisingly, and despite the fact that S-norketotifen was more potent than R-norketotifen in test of therapeutic activity, the S-enantiomer was less potent than RS-norketotifen and R-norketotifen in the present studies of antimuscarinic side effects.

3. Antihistaminic Activity *in vivo*.

The dorsal hair of male rats (150-200 g) were clipped and depilated. Animals were starved overnight and at least twelve hours after the depilation, the animals were orally pretreated with the test compound(s). Four dorsal test areas were marked with permanent ink, carefully avoiding the area closest to the spine. Exactly 60 min after the administration of the test compound, two intradermal injections of histamine (50 μL; 1.0 mg/ml of histamine di-HCl) were performed, one on each side on the back of the animal. Two intradermal injections of the vehicle for the histamine solution were also performed on each animal. Evans blue dye (20 mg/kg) was injected iv 1 min prior to pretreatment time expiration (= 1 min before the two intradermal injections of histamine and the two intradermal injections of the histamine vehicle). Twenty minutes were allowed for the wheal response to fully develop, whereupon the animals were euthanized through carbon dioxide asphyxiation. An incision was made along the spine and the dorsal skin containing the intradermal wheals was deflected. The blue spotted areas were measured in millimeters and the duplicate vehicle wheal responses were averaged. In vehicle-treated animals, the wheal area was on an average increased by histamine by 82 mm^2 (Study 1) and 94 mm^2 (Study 2), respectively. In animals pretreated with an active antihistamine compound, the histamine-induced response was less than that and the inhibition was calculated in %.

Two studies have been performed, one with the ketotifen isomers and one with the norketotifen isomers.

Study 1. Ketotifen Isomers.

	Dose (mg/kg)	Histamine response (mm^2)	Saline response (mm^2)	Difference (mm^2)	Inhibition (%)
Vehicle	---	107±4.1	25±1.0	82	---
R(+)ketotifen	0.1	94±7.2	22±2.6	72	12
R(+)ketotifen	1.0	68±2.0	18±1.2	50	39
R(+)ketotifen	10.0	28±2.2	24±0.0	4	95
S(–)ketotifen	0.1	87±6.7	22±2.0	65	21
S(–)ketotifen	1.0	70±8.0	21±1.9	49	40
S(–)ketotifen	10.0	28±2.2	22±2.0	6	93
RS(±)ketotifen	0.1	90±7.1	24±2.3	66	20
RS(±)ketotifen	1.0	68±6.5	21±1.9	47	43
RS(±)ketotifen	10.0	24±1.6	22±2.6	2	97
DPH	1.0	84	27	57	20
DPH	10.0	74	25	49	31

Conclusions: Both the racemate and the isomers of ketotifen are very potent antihistamines when tested in vivo. All three compounds were equipotent in this *in vivo* test. The results from a previous test of DPH (diphenhydramine; Benadryl®) is shown for comparison.

Results from Study 2 are shown on the next page.

Study 2. Norketotifen Isomers.

	Dose (mg/kg)	Histamine response (mm^2)	Saline response (mm^2)	Difference (mm^2)	Inhibition (%)
Vehicle	---	116±4.5	22±1.2	94	---
R(+)norketot	1.0	114±7.6	22±1.2	92	2
R(+)norketot	10.0	23±2.1	21±1.5	2	98
R(+)norketot	50.0	12±1.9	14±2.1	0	100
S(–)norketot	1.0	100±5.6	22±1.2	78	17
S(–)norketot	10.0	54±1.9	22±1.2	32	66
S(–)norketot	50.0	13±1.2	13±1.2	0	100
RS(±)norketot	1.0	114±3.9	22±1.2	92	2
RS(±)norketot	10.0	39±2.3	22±1.2	17	82
RS(±)norketot	50.0	10±1.2	12±0.9	0	100
DPH	1.0	84	27	57	20
DPH	10.0	74	25	49	31

Conclusions: Although less active than ketotifen, both the racemate and the isomers of norketotifen were very potent antihistamines when tested in vivo. Compare with (DPH) diphenhydramine, Benadryl®. R-, S-, and RS-norketotifen were equipotent in this study.

General Conclusion: Ketotifen is the most potent, commercially available H-1 blocker ever tested by us. Norketotifen was an effective antihistamine and significantly more potent than diphenhydramine (Benadryl®.)

4. Anti-inflammatory effects. (Inhibition of degranulation of human leukocytes)

Inhibition of stimulated histamine release from human leukocytes (buffy coat) by test articles was studied in these tests. The method used here was a modification of the methodology described by Nolte, H. and Stahl Skov, P.: Inhibition of basophil histamine release by methotrexate. Agents Action, 1988, 23: 173-176. In short, leukocytes were obtained from healthy volunteers and histamine release was induced by incubation (20 min/37°C) with A23187 (5 μM) in the presence or absence of a test article. Histamine was analyzed by enzyme-immuno assays (E.I.A.), using commercially available kits and a microplate reader (MRX, Dynatech). The test articles were tested in each assay at five concentrations ranging from $4x10^{-4}$ M to 10^{-6} M in duplicate to obtain inhibition curves.

In each experiment, the reference compound (cyclosporine) was tested in duplicate at eight concentrations to obtain an inhibition curve in order to validate the experiment.

The results were expressed as a percent of control values and as a percent inhibition of control values in the presence of the test articles. IC50 values (concentrations causing half-maximum inhibition of control values) were determined by non-linear regression analysis of the inhibition curves. These parameters were obtained by Hill Equation curve fitting. The IC50 values of the reference compound has passed the required inspections of the test facility and were within the limits of the historic average obtained ± 0.5 log unit.

After the development of the final test methodology, tests were performed with the racemates and the isomers of ketotifen and norketotifen: The first test compares the effects of ketotifen and norketotifen. The second test is a comparison between RS-, R(+)- and S(–)-ketotifen and the third test is a comparison between RS-, R(+)- and S(–)-norketotifen. Ketotifen and isomers were tested as fumarate salts, norketotifen and isomers were tested as hydrochlorides. IMPORTANT NOTICE: Since different blood donors were used in the three experiments, results cannot be compared between tests.

TEST 1. Comparison of ketotifen and norketotifen.

Test Article	IC50 (μM)
Ketotifen	91
Norketotifen	9.2
Cyclosporin A (reference compound)	0.11

Conclusion: Inhibition of histamine release is expressed as IC50 (μM). Norketotifen was approximately 10 times more potent than ketotifen in this test.

TEST 2. Comparison of RS-, R(+)- and S(–)-ketotifen.

Test Article	IC50 (μM)
RS-ketotifen	195
R(+)-ketotifen	>300
S(–)-ketotifen	131
Cyclosporin A (reference compound)	0.63

Conclusion: Inhibition of histamine release is expressed as IC50 (μM). S(–)-ketotifen was more potent than R(+)-ketotifen and RS-ketotifen in this test of anti-inflammatory activity..

The activity of RS-ketotifen was different in this test from TEST 1 (above), which can be expected since the blood samples were obtained from different individuals for the two tests; note that the results obtained with the reference compound were also different between the two tests.

TEST 3. Comparison of RS-, R(+)- and S(–)-norketotifen.

Test Article	IC50 (μM)
RS-norketotifen	58
R(+)-norketotifen	78
S(–)-norketotifen	39
Cyclosporin A (reference compound)	0.47

Conclusion: Inhibition of histamine release is expressed as IC50 (μM). S-norketotifen was twice as active as the corresponding R-isomer in this test. The activity of RS-norketotifen was different in this test from TEST 1 (above), which can be expected since the blood samples were obtained from different individuals for the various tests.

General Conclusion: It is clear from these studies that the anti-inflammatory activity of the drug ketotifen (Zaditen®) resides mainly in the active metabolite nor-ketotifen, not in ketotifen. S-norketotifen was surprisingly found to be twice as potent as the corresponding R-isomer and also more potent than racemic norketotifen. It was also surprisingly found that S-ketotifen was significantly more active than R-ketotifen.

5. Pulmonary Anti-inflammatory Activity

Male rats, 400-600 g, were injected i.p. with 10 μg PAF in 0.25% bovine serum albumin (BSA) in saline. Twenty-four hours later, the animals were sacrificed by i.p. injection of barbiturate and the trachea was exposed and cannulated. Aliquots (6 x 10 ml) of a buffered modified Tyrode solution were introduced successively and aspirated by gentle compression of the thorax. Total fluid recovery was usually above 80%. The cell suspensions were concentrated by low speed centrifugation (200 g for 10 min) and the resulting cell pellet was resuspended in 1 ml modified Tyrode solution. Total cell counts were made after diluting 10 μl of cell suspension in 90 μl of Turks fluid. Differential cell counts were made from smears fixed in methanol and stained with Leishman stain (only eosinophils are shown here). A total of at least 500 cells per smear were counted at 1000-fold magnification, in order to differentiate cell types. Drugs were administered for 7 days as a sustained subcutaneous infusion of 1.0 mg/kg/24hrs (Alza minipump). The exposure to PAF took place after five days of treatment with the test compound.

Results:

Test compound (1 mg/kg/day sc for 7 days)	N	PAF - induced eosinophilia (% ± SEM)
BSA + saline	8	100 ± 9
PAF + BSA +saline	10	250 ± 4
PAF + BSA + ketotifen	10	110 ± 3
PAF + BSA + norketotifen	10	74 ± 4

Conclusions:

Ketotifen reduced the eosinophil count from 250 % to 110 % while norketotifen reduced the eosinophil count to 74 %, which is significantly below the control value of 100 %. However, it has to be kept in mind that this is an in vivo study in rats and 70% of an oral dose of ketotifen is metabolized to norketotifen after oral dosing to rats (Morley, personal communication.) Thus, it can be concluded that norketotifen was significantly more potent than ketotifen as a pulmonary anti-inflammatory agent.

7. Sedative Activity Studies.

The physostigmine-induced lethality test used in BRIDGE PHARMA's studies is a modification of the sedation test technique that was used by Schering in the loratadine project (Villani F.J., et al. US Patent 4,659,716, 1987). In short, physostigmine (1.0 mg/kg s.c.) produces nearly 100% lethality when given to groups of mice (10 mice /group) confined to a small, well-defined volume of space. Mice administered a sedating drug prior to physostigmine administration are protected and survive. In the present study, test compounds are given orally 60 minutes prior to physostigmine injection. The number of sedated (surviving) mice was counted 30 min after the physostigmine dose.

(For Results, please see next page)

Conclusions:

The physostigmine-induced lethality test method used here has been extensively tested and validated. This test system has produced a perfect correlation to clinical experience, when used to test sedative side effects of antihistaminic drugs (Aberg et al., manuscript to be published.)

In the present test, ketotifen and diphenhydramine (Benadryl®) caused significant sedation. Surprisingly, S-ketotifen caused less sedation than R-ketotifen and RS-ketotifen. Thus, S-ketotifen will be an effective antihistaminic/anti-inflammatory treatment without the dose-limiting sedative side effect of racemic ketotifen (Zaditen®)

Surprisingly, norketotifen caused significantly less sedation than ketotifen. Thus, both RS- and R-norketotifen caused minor sedation, while S-norketotifen – surprisingly – was completely free from sedative effects.

The reference compound loratadine (Claritin®) was non-sedating, as expected.

Results:

	Oral Dose (mg/kg)	Sedated animals
RS-KETOTIFEN	100	9/10
RS-KETOTIFEN	150	10/10
R-KETOTIFEN	150	9/10
S-KETOTIFEN	150	3/10
RS-NORKETOTIFEN	100	0/10
RS-NORKETOTIFEN	150	1/10
S-NORKETOTIFEN	100	0/10
S-NORKETOTIFEN	150	0/10
R-NORKETOTIFEN	100	3/10
R-NORKETOTIFEN	150	3/10
VEHICLE	—	1/10
VEHICLE	—	0/10
LORATADINE(*)	150	1/10
DIPHENHYDRAMINE (*)	100	8/10

(*) Previous tests

Conclusions:

The physostigmine-induced lethality test method used here has been extensively tested and validated. This test system has produced a perfect correlation to clinical experience, when used to test sedative side effects of antihistaminic drugs (Aberg et al., manuscript to be published.)

In the present test, ketotifen and diphenhydramine (Benadryl®) caused significant sedation. S-ketotifen caused less sedation than R-ketotifen. Surprisingly, norketotifen caused much less sedation than the parent compound (ketotifen). R-norketotifen caused minor sedation and S-norketotifen – surprisingly – was completely free from sedative effects. The reference compound loratadine (Claritin®) was non-sedating.

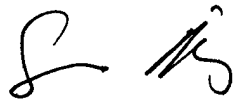
General Conclusions (Pharmacology)

Ketotifen (Zaditen®) is a potent antihistaminic and anti-inflammatory drug, but the severe sedative side effects of the drug limit the therapeutic dose to 1 or 2 mg. Ketotifen is metabolized in the liver and norketotifen is one of the metabolites that are formed. It has now surprisingly been found that after oral administration, the precursor ketotifen accounts for most of the sedation, while the metabolite norketotifen accounts for most of the anti-inflammatory activity. In light of the prior art (Polivka et al.), it was

unexpectedly found that S-ketotifen had clear advantages over R-ketotifen. Very surprisingly, S-norketotifen was found to be completely devoid of sedative side effects, although this enantiomer was at least as active and in several studies more active than R-norketotifen as an antihistaminic and anti-inflammatory agent. S-ketotifen and particularly S-norketotifen represent two of the extremely rare cases when a eutomer has both therapeutic and toxicological advantages over the corresponding distomer. These findings for the investigated S-enantiomers are particularly surprising, since the prior art (Polivka et al.) points in the opposite direction.

I further declare that all statements of the foregoing declaration made of my own knowledge are true and that those made upon information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statement may jeopardize the validity of the above-identified application or any patent issuing thereon.

Signed by me on this 6th day of June, 2003



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